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Investigation of Culture Filtrate of Endophytic Fungi *Nigrospora* sp. Isolate RS 10 in Different Concentrations towards Root-knot Nematode *Meloidogyne* spp.

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Abstract

One of the modes of action of endophytic fungi against plant pathogens e.g. plant parasitic nematode, is the production of toxic culture filtrate. Experiments were conducted to investigate the antagonistic effects of different concentrations of the culture filtrate produced by the endophytic fungi *Nigrospora* sp. isolate RS 10 against the root knot nematode *Meloidogyne* spp. *Nigrospora* sp. isolate RS 10 was isolated from the healthy root of a sengon plant (*Paraserianthes falcataria).* Three different concentrations of the culture filtrate were investigated using *in vitro* bioassays against the motile juvenile stage 2 of the root knot nematode *Meloidogyne* spp. Inactivation and mortality were recorded after 1, 3 and 24 hrs of exposure to culture filtrates. The mortality of the root-knot nematode *Meloidogyne*-J2 after 24 hrs exposure to the culture filtrate was confirmed by rinsing with sterile distilled water that demonstrated the effects of the culture filtrate treatment were irreversible, as nematodes did not recover. Nematode mortality rates after 1 to 24 hr exposure periods ranged from 80 % to 100 %. The effects of the culture filtrates on root-knot nematode *Meloidogyne*-J2 increased with increasing culture filtrate concentration.

Keywords: Endophytic Fungi, Culturfiltrate, Root-knot Nematode, *Meloidogyne* spp., *Nigrospora* sp.

1. Introduction

Plant parasitic nematodes are responsible for over $100 billion dollars in economic losses worldwide to a variety of crops. Root-knot nematodes are the most economically important group of plant parasitic nematodes worldwide, reducing both yield and crop quality [20, 12]. Infected plants show growth of reduction, swollen roots which develop into the typical root-knot galls, are two, or three times larger in diameter as healthy root. Root-knot nematodes are very difficult to control because they are polyphagous, where its over 2000 plants species is a highly specialized and complex feeding relationship with their host [6]. The life cycle is almost completely confined inside the host plant and high reproductive capacity. Although chemical control is still a common method for reducing nematode population, there is a considerable public pressure to limit or even ban the use of nematicides. Many nematicides are highly toxic and sometimes very mobile in the soil because of their solubility in water.

Concern over these chemicals has led to an increased interest in biological control in order to achieve more environmentally friendly methods of reducing nematode damage.

The term endophyte was coined by Heinrich Anton De Bary in 1884 and it is referred to fungi that colonize internal plant tissues without causing any apparent symptoms to the host plant [18, 20]. Fungal endophytes have been isolated from a great number of plants, such as bananas [14], maize [16], oilpalm [15], soybeans [8], potato [19, 25] and tomato [3]. Endophytes are most often isolated from symptomless plants of various species [10]. Their association with

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host plants is known to improve plant growth and vigor [26] and to potentially confer disease resistance in plants against pathogen infection [27].

The mechanisms of action responsible for nematode biocontrol mediated by endophytes are variable and include: the production of toxic secondary metabolites, competitive exclusion, competition for nutrients, predation, production of repellent compounds, alteration of root exudates, the induction of systemic resistance or a combination of these elements [1, 29, 25, 22, 7, 24].

2. Materials and Methods

2.1 Source of Endophytic Fungi *Nigrospora* sp. Isolate RS 10

Endophytic fungi *Nigrospora* sp. was originally isolated from cortical tissue of surface sterilized sengon root *P. falcataria* [17].

2.2 Preparation of Culture Filtrate

Pure fungal culture filtrate were obtained by centrifuging the fungal culture *Nigrospora* sp. isolate RS 10 in Czapek-Dox at 6000 rpm for 10 min [11]. The resultant supernatant was aseptically transferred to sterile 25 ml screw cap glass bottles and filtered through 0.22 µm millipore membrane filters. The pH of the fungal filtrates was measured and the average pH calculated. Sterile distilled water (H_2O) at pH ~7 and uninoculated Capek-Dox whose pH was adjusted to level of the average pH of the fungal culture-filtrates were the two controls in the experiment. Culture filtrates were kept in the fridge at 4°C overnight.

2.3 Source of *Meloidogyne* spp.

The root-knot nematode *Meloidogyne* spp. was originally isolated from an infested field on tomato plant in distric Barombong, South Sulawesi, Indonesia. The extraction of *Meloidogyne*-J2 was obtained by using the modified extraction technique of Hooper et al. [5]. Roots were washed free from soil under tap water, cut into 1 cm pieces and macerated in a warring blender at high speed for 20 s and collected in a glass bottle. Sodium hypochloride (NaOCl) was added to obtain a final concentration of 1.5% active Chlorine and the bottle was shaken vigorously for 3 min. The suspension was then thoroughly washed with tap water through a sieve combination 250, 100, 45 and 25 µm mesh to remove the NaOCl. Eggs were collected on the 25 µm sieve and then transferred to a glass bottle. The egg suspension was supplied with oxygen from an aquarium pump over 10 days to induce juvenile hatching. To separate active J2 from unhatched eggs or dead J2, a modified Baermann technique over 24 hours was used. The collected active J2 were adjusted to 1000 J2 5 ml-1 and used immediately as source of inoculum.

2.4 Investigation of Culture Filtrate *Nigrospora* sp. isolate RS 10 on Inactivation and Mortality of *Meloidogyne*-J2

One milliliter of 2-wk-old undiluted culture filtrates transferred to 30-mm-diameter sterile glass petri dishes. The filtrates were inoculated with approximately 100 nematode of *Meloidogyne*-J2. Control petri dishes had pure Czapek-Dox inoculated with the nematode of *Meloidogyne*-J2. The investigation was laid in a completely randomized design on a laboratory bench. All investigation were repeated thrice over time, with three replicates per concentration. Inactivation and Mortality was determined by counting the number of active, inactive and dead nematode of *Meloidogyne*-J2 after 1, 3 and 24 hours of exposure to culture filtrate. Individual counts of each category were recorded for each time interval. The activity (mobility) of nematodes immersed in diluted culture filtrates was determined by counting the number of active and inactive nematodes after an exposure time of 1, 3 and 24 hrs. After 24 hours, nematodes from each replication were rinsed with sterile distilled water after concentrating them using 28-µm sieve and transferring them back into the petri dishes containing sterile distilled water. The petri dishes were left on the bench under laboratory conditions for extra 24 hours. Nematodes were probed with a fine needle under the microscope and those which were straight in shape and remained immotile even after probing were considered dead [14].

2.5 Data Analysis

Abott's corrected mortality formula was used to calculate nematode inactivation. Mortality was calculated as the percentage of mortalited nematodes out of the total original number of nematodes, at each time interval. Corrected inactivation and percentage mortality data was used to evaluate the effects of individual concentrations. Data were arcsine-square root transformed before analysis of variance (ANOVA). Laverne test was used to test for normality

of distribution and homogeneity of variances. Analysis of Variance was carried to determine single factor effects and factor interactions. Where evident, effects of one factor were analysed at each time of the interacting factor. Effects of the factors were significant, means were separated using the Tukey's test.

3. Result and Discussion

3.1 Result

The percentage of inactivation nematodes by culture filtrates of *Nigrospora* sp. isolate RS 10 increased with an increase in filtrate concentration (Table 1). Differences in the percentage of inactivation nematodes as the culture filtrate concentration increased from 25% to 100% and significant difference (P<0.05). Culture filtrate concentration of 100% in 3 and 24 hours exposure reached 100% inactivation of nematode *Meloidogyne*-J2.

Percentage mortality of nematodes differed significantly among the different culture filtrate concentrations (P<0.005), and increased as the filtrate concentration increased (Figure 1). Significant differences in the percentage of dead nematodes were evident all of filtrate concentrations. In the culture filtrate of 100% and 24 hours exposure, followed by rinsing with distilled water still 100% nematode *Meloidogyne*-J2 dead.

3.2 Discussion

With increasing concentrations of culture filtrates of *Nigrospora* sp. isolates RS 10 increased the inacvtivation and the mortality of the nematodes. Culture filtrate at concentrations of 100% and 50% could be measured, a nearly 100% inactivation of the nematodes. Nur Amin [14] also noted a tendency depended the inactivation of the concentration at which he has examined *Fusarium oxysporum* A1 culture filtrates against burrowing nematode *Radopholus similis*. The same result reported Schuster [12] that inactivation of the nematode *P. redivivus* and *G. Pallida* depending on culture filtrate concentration. Cayrol and Djian [2] found in the culture filtrates of them studied by *F. roseum* var arthtosporioides at the concentrations of 50% and 100% after 24 hours of contact time a 100% inactivation of *M. arenaria*-J2.

Hayashi et al. [4] also confirmed that a depending of the effect culture filtrate concentration of *Irpex lacteus* against the nematode *Aphelenchoides besseyi*. Even with Molina and Davide [13] the effect of the concentration was examined at 4 various fungal culture filtrates. Thus, at the concentrations of 50 ppm or 200 ppm of the culture filtrate of *Penicillium oxalicum* with *M. incognita* a mortality was determined by 13.5% and 78.6%. For *Meloidogyne*-J2 in this investigation, a death rate of 45% and 100% respectively.

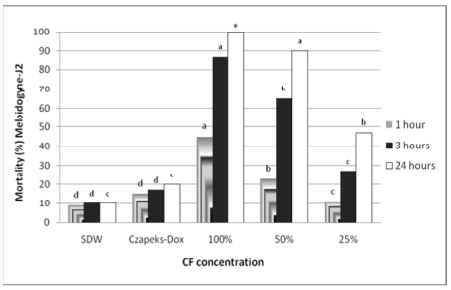
Table 1. Percentage Inactivation of *Meloidogyne*-J2 in Different Culture filtrate Concentrations of Endophytic *Nigrospora* sp. isolate RS10 after 1, 3 and 24 hours-exposure

CF Concentration	Hours after Treatments Inactivation(%)*Meloidogyne*-J2		
	1	3	24
SDW	7	7	10
Czapeks-Dox	12	14	18
100 %	80[a]	100[*]	100[*]
50 %	61[b]	80[a]	95[a]
25 %	52[c]	67[b]	84[b]

Means followed by the same letter (superscript) for each isolate in each bioassay are not significantly different at P = 0.05 according to Duncan's Multiple Range Test, n = 3; Czapeks-Dox as control media; *values could not be statistically calculated.

The use of diluted culture filtrates was intended to mimic the natural conditions encountered by root-knot *Meloidogyne* spp. in their host plants. As expected, the percentage immobilized nematodes decreased with a reduction in culture filtrate concentration. This is in agreement with findings by others regarding the effects of fungal filtrate concentrations on the immobilization of nematodes [9, 30]. The low rates of nematode immobilization at low culture filtrate concentration might be due to the dilution of the toxic compounds. If the fungal isolates produce the toxins inside the plant, that concentration would probably be lower than in the bioassays conducted with undiluted culture filtrates. Thus, the levels of nematode control in the plants due to toxins can be expected to be lower than in the laboratory.

Production of toxins in the plant may, however, act as an additional form of armory that would help the plant guard it self from invasion by the nematode. Production of toxic metabolites in the plant by endophytes fungi *Fusarium* may inhibit not only nematode mobility, but also host searching and the infection processes [23]. While the production of toxic metabolites may be the main mechanism of action in the laboratory, the value of nematicidal compounds in the plant, and the mechanism where by endophytes protect host plants against root-knot nematode should be further investigated.



The numbers followed by the same letter (superscript) for each isolate in each bioassay are not significantly different at P = 0.05 according to Duncan's Multiple Range Test, n = 3; Czapeks-Dox as control media; * values could not be statistically calculated.

Figure 1. Percentage Mortality of *Meloidogyne*-J2 in Different Culture filtrate Concentrations of Endophytic *Nigrospora* sp. Isolate RS10 after 1, 3 and 24 hours exposure.

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